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(71) Applicant: HITACHI, LTD.
6, Kanda Surugadai 4-chome
Chiyoda-ku, Tokyo 100(JP)

(72) Inventor: Takeuchi, Kouji
5-329, 3-290-1 Matsugaoka
Nagareyama-shi, Chiba(JP)
Inventor: Kawaguchi, Hideo
C-204, 2-32, Koyasicho
Hachioji-shi, Tokyo(JP)
Inventor: Ishibashi, Tadashi
5-17-1-2, Jousuihoncho
Kodaira-shi, Tokyo(JP)
Inventor: Shimizu, Norio
4-6-2 Honcho
Kokubunji-shi, Tokyo(JP)

(74) Representative: Patentanwälte Beetz sen. -
Beetz Jun. Timpe - Siegfried -
Schmitt-Fumian-Mayr
Steinsdorferstrasse 10
D-8000 München 22(DE)

(54) Method and apparatus for controlling cultivation conditions for animal cells.

(57) The invention relates to a method and an apparatus for cultivating animal cells. Cultures of animal cells stable for a long time are accomplished by calculating the ratio (L/G) of the amount of lactic acid formed by the cultivation of the animal cells to the amount of glucose consumed thereby, within a pre-determined time period determining the growth conditions of the animal cells therefrom and thus controlling the cultivation conditions. When the ratio L/G, which is preferably from greater than 0 to 0.6, is outside the desired range, the dissolved oxygen concentration and/or the glucose concentration in the culture medium may be controlled by increasing the dissolved oxygen concentration in the culture medium and/or decreasing the glucose concentration therein.

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Method and Apparatus for Controlling Cultivation Conditions for Animal Cells

This invention relates to a method and an apparatus for controlling cultivation conditions for animal cells.

Animal cells can produce proteins which cannot be produced by recombinant microorganisms, for example, those to which oligosaccharides are bound, those having high molecular weights and those having complicated stereochemical structures. Therefore, it has attracted scientific attention to produce these proteins by culturing animal cells. In particular, there has been an urgent demand to provide a process for the mass production of animal cells.

Technical problems observed in the high-density cultivation of cells involve the removal of proliferation inhibiting factors such as lactic acid and ammonia which are metabolites secreted by the cells. It is known that lactic acid and ammonia inhibit the proliferation of cells (see T. Kimura et al., Journal of Fermentation Technology, 65 (3), 341-344 (1987)). Thus there has been proposed a process for perfusion culture wherein fresh medium is continuously supplied while used medium is withdrawn so as to remove these proliferation inhibiting factors (see JP-A-36915/1986 and JP-A-257181/1986). In this perfusion cultivation process, the rates of feeding the fresh medium and withdrawing the used one are controlled mainly based on the proliferation rate of the cells. Thus it has been a practice that when this rate decreases, the rates of feeding the fresh medium and withdrawing the used one are increased so as to dilute the proliferation inhibiting factors (see M. Tokashiki et al., Kagaku Kogaku Ronbunshu, 14 (3), 337-341 (1988)).

The above mentioned prior art, wherein the proliferation inhibiting factors are diluted by feeding the medium, is disadvantageous in that the economic efficiency of the substrate-containing medium is not considered and thus the consumption of a large amount of substrate-containing medium elevates the running cost.

The present inventors have found out that the lactic acid concentration in a culture medium of animal cells is indicative for the proliferation status of the cells, and the lactic acid concentration can be lowered by nutritionally starving the animal cells and thus allowing the animal cells to assimilate the lactic acid accumulated in the culture medium.

The term animal cells includes mammalian and particularly human cells.

Conventional microorganism culture systems can hardly be applied to the mass production of animal cells for the following reason. An animal cell has no cell wall and thus shows a poor resistance

against shearing forces caused by stirring. Serum contained in a culture medium would lead to foaming during aeration. The long generation time of animal cells requires maintaining the system axenic for several tens of hours. It is particularly required to maintain a sensor for measuring the dissolved oxygen concentration in the culture medium stable for a long time, since the cultivation should be continued for a long time. However, an oxygen sensor would be damaged by heat or a change in pressure during steam sterilization. Further, the prolonged use of an oxygen sensor would sometimes cause drying up of liquid in the sensor or formation of a protein membrane at the sensor/liquid interface, which make the measurement impossible. When these troubles make the measurement of the dissolved oxygen concentration impossible, the controlled cultivation cannot be continued any more. Excessive supply of oxygen would be toxic for the animal cells, while shortage of oxygen would lower the proliferation.

In addition, there are no known optimum cultivation conditions for animal cells.

The failure in the measurement of the dissolved oxygen concentration and the lack of any procedure for determining the growth activity of animal cells makes it difficult to continue the cultivation for a prolonged period of time.

Accordingly, it is the object of the present invention to provide a method and an apparatus for controlling cultivation conditions for animal cells which allow the high-density cultivation of animal cells and whereby the concentration of proliferation inhibiting factors can be efficiently decreased while efficiently utilizing the substrate-containing medium so that animal cells can be stably cultured for a long time.

The above mentioned object is achieved according to the claims. The dependent claims relate to preferred embodiments.

The method according to the present invention for controlling the cultivation conditions for animal cells in a culture medium containing a substrate is characterized by

- 45 - measuring the lactic acid concentration c_l in the culture medium of the animal cell, and
- controlling the dissolved oxygen concentration and/or the substrate concentration c_s in the culture medium in accordance with the measured lactic acid concentration c_l .

In accordance with a preferred embodiment, the method of the present invention is characterized by

- measuring the lactic acid concentration c_l in the culture medium of the animal cells,

- measuring the substrate concentration c_s of the culture medium of the animal cells,
- determining the concentration L of lactic acid formed in a predetermined time period,
- determining the concentration S of the substrate consumed in that predetermined time period,
- calculating the ratio L/S, and
- controlling the dissolved oxygen concentration and/or the substrate concentration c_s in the culture medium in accordance with the ratio L/S.

In particular, the method of this invention is characterized by

- measuring the lactic acid concentration c_l and the substrate concentration c_s of the culture medium of the animal cells at predetermined times defining predetermined time periods Δt or continuously,
- calculating the concentration L of lactic acid formed during the time period Δt between subsequent predetermined times or continuously,
- calculating the concentration S of substrate consumed during the same predetermined time periods Δt ,
- calculating the respective ratio L/S,
- and
- controlling the dissolved oxygen concentration and/or the substrate concentration c_s in the culture medium such as to keep the ratio L/S within a predetermined, fixed range.

According to a preferred embodiment, the dissolved oxygen concentration and/or the substrate concentration (c_s) in the culture medium is performed when the ratio L/S is or tends to be outside the predetermined, fixed range:

In the case of discontinuous measurement or detection of the lactic acid concentration c_l and the substrate concentration c_s at predetermined times defining predetermined time periods Δt , L and S are defined as follows:

$$L \approx \left| \frac{\Delta c_l}{\Delta t} \right|, \text{ and}$$

$$S \approx \left| \frac{\Delta c_s}{\Delta t} \right|,$$

L representing the rate of lactic acid formation, and S representing the rate of substrate consumption, based on the same times or time periods Δt .

In accordance therewith, the ratio L/S is defined as follows:

$$5 \quad L/S \approx \frac{\left| \frac{\Delta c_l}{\Delta t} \right|}{\left| \frac{\Delta c_s}{\Delta t} \right|} \approx \left| \frac{\Delta c_l}{\Delta c_s} \right|.$$

10 In the case of continuous measurement of the lactic acid concentration c_l and the substrate concentration c_s , the differential quotients

$$15 \quad L = \frac{dc_l}{dt} \text{ and } S = \frac{dc_s}{dt}$$

are determined, and the ratio L/S is calculated according to

$$20 \quad L/S = \frac{\left| \frac{dc_l}{dt} \right|}{\left| \frac{dc_s}{dt} \right|} = \left| \frac{\frac{dc_l}{dt}}{\frac{dc_s}{dt}} \right|.$$

30 In case glucose is used as the substrate, the glucose concentration c_g is determined in the same manner as defined above for the substrate concentration c_s , and accordingly, the concentration G of glucose consumed in said predetermined time periods Δt , or the glucose consumption rate, is

$$35 \quad G \approx \left| \frac{\Delta c_g}{\Delta t} \right| = \left| \frac{dc_g}{dt} \right|,$$

40 and the ratio L/S corresponds in this case to

$$45 \quad L/G \approx \frac{\left| \frac{dc_l}{dt} \right|}{\left| \frac{dc_g}{dt} \right|} = \left| \frac{\frac{dc_l}{dt}}{\frac{dc_g}{dt}} \right|.$$

50 In the case of a constant lactic acid formation rate and a constant substrate (glucose) consumption rate,

$$55 \quad \left| \frac{\Delta c_l}{\Delta t} \right| = \left| \frac{dc_l}{dt} \right|, \text{ and}$$

$$\left| \frac{\Delta c_s}{\Delta t} \right| = \left| \frac{dc_s}{dt} \right| \quad (\left| \frac{dc_g}{dt} \right| = \left| \frac{dc_g}{dt} \right|),$$

and accordingly, L/S is in this case

$$L/S = \frac{\Delta c_1}{\Delta c_s},$$

and L/G is

$$L/G = \frac{\Delta c_1}{\Delta c_g}.$$

In accordance with a preferred embodiment, the dissolved oxygen concentration and/or the substrate concentration c_s (c_g) are controlled by stepwise supplying oxygen and/or substrate (glucose) to the culture medium at predetermined times, and the ratio L/S (L/G) is determined for each time period Δt between these predetermined times.

The predetermined, fixed range of the ratio L/S (L/G) is defined as to be

$$0 < L/S (L/G) \leq A,$$

A being an upper limit value particularly depending on the kind of the cultivated animal cells, and preferably being 0.6.

By means of these measures, the lactic acid concentration in the culture medium can be controlled and kept below a predetermined value.

That controlling is preferably performed by increasing the dissolved oxygen concentration and/or by decreasing the substrate concentration c_s - (glucose) concentration c_g in the culture medium.

It may be preferred to supply oxygen to the culture medium continuously for the entire culturing time.

In accordance with a preferred embodiment of the present method, the animal cells are periodically separated from the culture medium and put into a new culture medium, preferably into a new culture medium having a higher substrate/glucose concentration than the used culture medium; this is repeatedly carried out during the entire culturing time.

The lactic acid concentration in the culture medium can be lowered e.g. by temporarily starving the animal cells nutritionally and thus allowing the animal cells to assimilate lactic acid secreted by the animal cells to thereby control the lactic acid concentration in the culture medium to be below a predetermined value, preferably below 2.5 g/l.

In the above mentioned cultivation process, cells capable of expressing a gene of an enzyme which assimilates lactic acid may be used as the animal cells. It is preferable to use cardiac type lactate dehydrogenase as the enzyme which as-

similates lactic acid.

Further, cells originating from liver, and more particularly, cells of the rat ascites hepatoma cell JTC-1 strain may be used. This strain which is described in Journal of Experimental Medicine, 28 (2), 115-117 (1858) and has been authorized by the Japanese Society of Tissue Culture, can be readily obtained.

In the cultivation process of the present invention, the predetermined value of the lactic acid concentration may vary depending on, for example, the animal cells to be used, the D cell concentration in the cultivation, and the kind of the medium.

Reducing the substrate/glucose concentration leads to a reduction of the cell growth and induces lactic acid assimilation when the lactic acid concentration c_l of the culture medium reaches a predetermined value. This is performed preferably when the lactic acid concentration c_l reaches 2.5 g/l. In this connection, it is preferable to add, throughout the entire cultivation, substrate/glucose until the lactic acid concentration c_l reaches the predetermined value.

The reduction of the substrate/glucose concentration is advantageously performed by controlling or by stopping the addition of substrate/glucose.

Further, the invention provides an apparatus for controlling cultivation conditions for animal cells in a culture medium containing a substrate which is particularly suited for carrying out the above described method; the apparatus comprises

- a fermentor for the culture medium, preferably provided with a stirrer,
- a substrate tank connected to the fermentor by means of a conduit comprising a control valve,
- lactic acid concentration sensing means provided at or in the fermentor, and
- an operation controller for controlling the control valve and receiving the output signal of the lactic acid concentration sensing means. Control may be effected on the basis of a reference value for the lactic acid concentration.

Optionally, the apparatus comprises a cell/culture medium separator provided at the discharge side of the fermentor and a culture medium supernatant pump provided in the discharge conduit of the fermentor.

In accordance with another preferred embodiment, this apparatus further comprises

- means for supplying oxygen to the fermentor, and
- glucose concentration sensing means provided at or in the fermentor and connected to the operation controller.

The oxygen supply means advantageously comprise an oxygen supply conduit and a control

valve provided therein and being controlled by the operation controller.

In accordance with another preferred embodiment, the operation controller is adapted to carry out the above described variants of the method of this invention by controlling the supply of oxygen and/or of substrate by means of the substrate control valve and/or the oxygen control valve, and eventually the discharge of the culture medium and the separation of the culture medium supernatant by means of the cell/culture medium separator, on the basis of the output signals of the lactic acid concentration sensing means and eventually also the glucose concentration sensing means.

The operation controller calculates the ratio L/S (L/G) based on the sensor input data and compares the ratio thus calculated with the fixed range which has been preliminarily input, and the dissolved oxygen concentration and/or the glucose concentration in the culture medium is controlled based on the result of this comparison.

The features and advantages of the present invention will become more clear from the following detailed description of preferred embodiments shown in the drawings, wherein:

Figs. 1(a) and 1(b), 2(a) and 2(b), 3(a) and 3(b), 4(a) and 4(b), and 5(a) and 5(b) show experimental results of animal cell cultivation examples;

Fig. 6 is a schematic view of an apparatus of the present invention;

Figs. 7(a) and 7(b) show results of an example of the present invention;

Figs. 8(a) - 8(c) are graphs showing that the JTC-1 strain will assimilate lactic acid;

Fig. 9 is another example of an apparatus of the present invention; and

Figs. 10(a) - 10(c) are graphs showing the results of using JTC-1 strain with the apparatus of Fig. 9, using lactic acid as an indicator.

Under aerobic conditions, animal cells acquire energy by converting glucose into carbon dioxide and water via the tricarboxylic acid cycle. The use of the tricarboxylic acid cycle is suitable for the growth of animal cells, and 38 mol of adenosine triphosphate (ATP) are formed from 1 mol of glucose in this cycle. Under conditions where oxygen is insufficient, on the other hand, animal cells acquire energy by converting glucose into lactic acid via the glycolysis pathway depending on the Pasteur effect. In this latter case, only two mol of ATP can be formed from one mol of glucose. Thus the exclusive use of the glycolysis pathway results in an extremely low efficiency of the utilization of glucose. The conditions of the supply of oxygen and the growth activity of the cells are determined by using the ratio of the formed lactic acid to the consumed glucose (L/G) as an indication.

Even though a sufficient amount of oxygen is

supplied, furthermore, lactic acid would be formed by Crabtree's effect when a culture medium contains glucose at a high concentration (cf. Crabtree, Biochemistry 23, 537-545 (1929)). In this case, the cells acquire energy by using the glycolysis pathway exclusively. Thus the growth activity of the cells is low.

The growth activity of cells is determined by measuring the glucose concentration and lactic acid concentration in the culture medium and using the ratio of formed lactic acid to consumed glucose (L/G) obtained from these data as an indication to thereby get an information about the cultivation conditions and control the same.

The preferred range of the L/G ratio has been established through the following cultivation experiments conducted under various conditions, and is found to be $0 < L/G \leq 0.8$.

An air filter (mid. by Gelman Science, 7.5 cm^2) was attached to the lid of a 200-ml taping culture flask in such a manner that an oxygen-containing gas stream was introduced therethrough into the container. 80 ml of a DM160-AU medium (mid. by Kyokuto Seiyaku Kogyo K.K.) containing 10 % of new born bovine serum was fed into the container and then inoculated with seed cells of JTC-1 strain (rat abdominal hepatoma cells) at a concentration of $1 \times 10^6 \text{ cells/ml}$. Then the cells were cultured with stirring at 400 min^{-1} at 37°C and with use of a CO_2 incubator. The culture medium was centrifuged at 1200 min^{-1} for 10 min every two days, and the centrifugally separated cells were placed in a new medium, which corresponds to an exchange of the whole medium. The initial glucose concentration of the culture medium was adjusted to 3500 mg/l at the time of each exchange. Before and after each exchange of the whole medium, 2-ml portions of the culture medium were sampled, and the glucose concentration and lactic acid concentration of each sample was measured with an enzyme sensor. Further the cell concentration was measured with a hemacytometer. Fig. 1 shows the results obtained. The cells grew well, and the cell concentration reached $1 \times 10^7 \text{ cells/ml}$ on the tenth day. The ratio L/G was as high as 0.5 at the early stage of the cultivation, and then slowly decreased and finally reached 0.3. Thus it was found that a ratio L/G of 0.8 or below indicated that the cultivation conditions were aerobic and a sufficient amount of glucose was supplied, i.e., the cells showed a high growth activity and the optimum cultivation conditions were established.

In the following examples, the conditions applied are the same except where differences are noted.

Next, the glucose concentration in the culture medium at the time of each exchange of the whole medium was adjusted to 1000 mg/l, and the air

filter was removed. Namely, the cultivation was carried out under hermetically sealed conditions. Fig. 2 shows the results obtained. The final cell concentration was 5.5×10^6 cells/ml while the ratio L/G exceeded 0.6 throughout most of the cultivation period. Thus it was found that the growth activity of the cells was lowered when the ratio L/G exceeded 0.6 and the glucose concentration was not excessively high.

Next, the glucose concentration in the culture medium at the time of each exchange of the whole medium was adjusted to 1000 mg/l, and the air filter was attached to the lid. Fig. 3 shows the results. The final cell concentration was 7.5×10^6 cells/ml while the ratio L/G decreased with the lapse of time and reached zero on the eighth day simultaneously with the consumption of the glucose. Thus it was found that the glucose concentration of 1000 mg/l was insufficient for achieving a high-density cell concentration of 1×10^7 cells/ml or higher.

Then mouse hybridoma 11D-11-1 strain (parent strain: P3-U1) was cultured. An air filter was attached to the same tapping culture flask as the one described above to thereby introduce an oxygen-containing gas stream with variable oxygen concentrations into the container. 80 ml of a serum-free medium E-RDF + RD-1 (mfd. by Kyokuto Seiyaku Kogyo K.K.) was fed into the container and inoculated with seed cells 11D-11-1 at a concentration of 1×10^6 cells/ml. Then the cells were cultured with stirring at 300 min^{-1} at 37°C with use of a CO_2 incubator. The culture medium was then centrifuged at 1200 min^{-1} for 10 min everyday to thereby exchange the whole medium. The initial glucose concentration of the culture medium was adjusted to 3423 mg/l at the time of each exchange. Before and after each exchange of the whole medium, 2-ml portions of the culture medium were sampled, and the glucose concentration and lactic acid concentration of each sample was measured with an enzyme sensor. Further the cell concentration was measured with a hemacytometer. Fig. 4 shows the obtained results. On the fourth day of the cultivation, the cell concentration reached 3×10^6 cells/ml, and the growth ceased when the L/G ratio exceeded 0.6. When the oxygen concentration of the oxygen-containing gas was adjusted to 60 %, however, the cell concentration reached 5×10^6 cells/ml, and the L/G ratio decreased to 0.4 on the seventh day. Thus it was found that a L/G ratio of 0.6 or below indicated that the cultivation was conducted under aerobic conditions and the cells showed a high growth activity, namely, conditions suitable for the growth were established.

Next, the cultivation was conducted with an oxygen concentration of the oxygen containing gas

which was constantly kept at 20 %. Fig. 5 shows the results. The cell concentration reached 3×10^6 cells/ml on the third day but the cells did not grow any more. The L/G ratio exceeded 0.8 throughout the cultivation period. Thus it was found that the supply of oxygen was insufficient and the growth activity of the cells was lowered when the L/G ratio exceeded 0.6 and the glucose concentration was not excessively high.

The results of these Experimental Examples show that the growth conditions of cells can be determined from the consumed glucose and the formed lactic acid, particularly their ratio. The cells particularly grew well when the L/G ratio ranged from greater than zero to 0.6, which suggests that the supply of glucose and oxygen under such conditions might be suitable for the growth of the cells.

In the method for controlling cultivation conditions according to the present invention, therefore, the dissolved oxygen concentration and/or glucose concentration in the culture medium should be controlled when the L/G is outside a fixed range, which preferably is from greater than 0 to 0.6.

The control may be conducted by increasing the dissolved oxygen concentration of the culture medium and/or decreasing the glucose concentration thereof. When the L/G ratio exceeds 0.6, for example, the supply of oxygen is first increased, and it is examined whether the L/G ratio is lowered to 0.6. When the L/G ratio is still above 0.6, the control can be achieved by decreasing the supply of glucose.

An example of a cultivation with controlling according to the present invention will be described by reference to Fig. 6. A fermentor 1 contains a culture medium 2 in which cells may be optionally immobilized, for example, on a microcarrier. The cells are suspended in the culture medium 2 by a stirrer 4 comprising rotating stirring blades with use of a motor 3. Oxygen may be supplied by either overlay aeration, bubbling or membrane aeration. The oxygen-containing gas is fed into the fermentor 1 through a conduit 13, and the O_2 flow rate is controlled with a control valve 6. Preserve culture medium containing glucose or an aqueous solution of glucose is provided in a substrate tank 5. The glucose-containing solution is fed to the fermentor 1 through a conduit 14, and the flow rate thereof is controlled with a control valve 7. The cultivation may be conducted by, for example, semibatch culture or continuous culture. Continuous culture is suitable for mass production. The culture supernatant is separated from the cells by a cell/culture medium separator 10 and discharged out of the fermentor 1 through a discharge conduit 17 with use of a culture supernatant pump 11. The con-

centrations of lactic acid and glucose in the culture medium 2 may be measured by, for example, using an enzyme sensor or liquid chromatography. In this case, they are measured with a glucose concentration sensor 8 and a lactic acid concentration sensor 9, respectively, which are connected to the fermentor 1 by means of conduits 15 and 16, respectively. The output data from the sensors 8, 9 are input to an operation controller 12 where calculations are performed and control signals are generated according to the ratio L/G. Then the control signals are output to the control valves 6 and 7 to control the supply of oxygen and substrate, respectively.

The present invention is based on the finding that the ratio of the concentration of lactic acid formed in the culture medium to that of the consumed glucose (L/G) correlates to the dissolved oxygen concentration in the culture medium and to the growth conditions of the cells. Further, it had been found that the cells grew well when the L/G ratio was within a fixed range, in particular, from greater than 0 to 0.6. Accordingly, the cultivation conditions for animal cells may be appropriately controlled by maintaining the L/G ratio within that range. In the present invention, the ratio L/G is maintained within the predetermined range by controlling the dissolved oxygen concentration and/or the glucose concentration.

The present invention is further illustrated by way of the following example.

An air filter (mfd. by Geiman Science, 7.5 cm²) was attached to the lid of a 200-ml tapping culture flask in such a manner that an oxygen-containing gas stream was introduced through the filter into the container. 80 ml of a DM160-AU medium (mfd. by Kyokuto Seiyaku Kogyo K.K.) containing 10 % of new born bovine serum was fed into the container and then inoculated with seed cells of JTC-1 strain (rat abdominal hepatoma cells) at a concentration of 1×10^6 cells/ml. Then the cells were cultured with stirring at 400 min⁻¹ at 37 °C, and with use of a CO₂ incubator. The culture medium was centrifuged at 1200 min⁻¹ for 10 min everyday to thereby exchange the whole medium.

Before and after each exchange of the whole medium, 2-ml portions of the culture medium were sampled, and the glucose concentration and the lactic acid concentration of each sample were measured with an enzyme sensor. Further the cell concentration was measured with a hemacytometer. The glucose concentration of the medium to be exchanged was determined according to an assumed glucose consumption rate of the JTC strain of 1×10^7 mg/cell/d. When the L/G ratio exceeded 0.6, this value was decreased. When the L/G ratio fell down to 0.4 or below, on the other hand, this value was increased for safety. Fig. 7

shows the results of the cultivation. The cell concentration reached 1×10^7 cells/ml on the fifth day. The L/G ratio was high on the first day, slowly decreased thereafter and reached 0.4 on the third day. Then the supply of glucose was increased, which caused an increase in the L/G ratio. Except for the first day, the L/G ratio was within the range of from 0 to 0.6, and the cells grew well. Thus a high density cultivation of 1×10^7 cells/ml was effected.

The lactic acid concentration may be controlled to be below a predetermined value by controlling the medium, particularly the flow rate of the substrate depending on the lactic acid concentration in the culture medium in, for example, the following manner with respect to Figures 8 and 10.

After initiation of the cultivation, the substrate is continuously added while the lactic acid concentration is lower than the predetermined value. The rate of feeding the substrate may be increased. When the lactic acid concentration exceeds a predetermined value, the addition of the substrate is ceased or the rate of its addition is lowered. Thus the lactic acid is assimilated by the animal cells, which lowers the lactic acid concentration.

Although the predetermined value of the lactic acid concentration may vary somewhat depending on the kind and concentration of the animal cells and the kind of the medium, it is generally preferable to be below 2.5 g/l.

Figs. 8(a) - 8(c) show the results of the cultivation of JTC-1 strain. 80 ml of a DM-160AU medium (mfd. by Kyokuto Seiyaku Kogyo K.K.) containing 10 % of new born bovine serum was fed into a 200-ml tapping culture flask and then inoculated with seed cells of JTC-1 strain at a concentration of 4×10^5 cells/ml. Then the cells were cultured while stirring at 400 min⁻¹ and 37 °C in an incubator. After 1.6 and 3.2 days, the culture medium was centrifuged at 12,000 min⁻¹ for 10 min to separate the cells from the used medium; then, new medium was added to the cells, thereby exchanging the whole medium. 2-ml portions of the culture medium were sampled everyday to measure the glucose concentration and lactic acid concentration of each sample with an enzyme sensor. Further the cell concentration was measured with a hemacytometer. As a result, the cell concentration on the fourth day was found to be 4×10^6 cells/ml, showing an excellent growth. The glucose concentration decreased to 0 mg/l after 1.6, 2.4, and 4.1 days, and an obvious decrease in the lactic acid concentration was observed each time.

These results indicate that the cells used above obviously assimilate lactic acid when glucose used as the substrate is running out. Therefore, the assimilation of lactic acid may be appropriately effected by measuring the lactic acid con-

centration in the culture medium and decreasing the amount of the substrate to be added or stopping the addition of the substrate, when the lactic acid concentration exceeds the predetermined value as a way of controlling the medium, particularly adjusting the glucose concentration. When the lactic acid concentration falls below the predetermined value again, the amount of the substrate to be added may be increased, or the addition of the substrate may be started again.

The term "substrate" as used herein means a carbon source required in the cultivation of the animal cells, and examples thereof include glucose, fructose and galactose. The substrate may be either a solid or a liquid. A liquid substrate may be mixed in water or in another medium.

Now an example of the cultivation apparatus of the present invention will be described by reference to Fig. 9 where the same reference numerals designate the same elements as in Fig. 8. The fermentor 1 contains a culture medium 2 in which cells, which may be optionally immobilized on, for example, a microcarrier, are suspended. The cells are suspended by means of a stirrer 4 comprising rotating stirring blades and a motor 3. Oxygen may be supplied by either overlay aeration, bubbling or membrane aeration. A medium containing glucose dissolved therein or an aqueous solution of glucose is stored in the substrate tank 5. A solution containing substrate is fed to the fermentor 1 via the conduit 14, and the feed rate is controlled with the control valve 7. The cultivation may be conducted by, for example, semi-batch culture or continuous culture, of which the continuous culture is suitable for mass production. The culture supernatant is separated from the cells with the cell/culture medium separator 10 through filtration or sedimentation and withdrawn out of the fermentor 1 through the conduit 17 with the use of the culture medium supernatant pump 11. The lactic acid concentration in the culture medium 2 may be measured by, for example, using liquid chromatography or with an enzyme sensor as lactic acid concentration sensor 9 connected to the fermentor 1 through the conduit 16. The data thus obtained are input into the operation controller 12 for comparison and judgment, and then control signals are output to the control valve 7 to control the feed rate of the substrate. When the lactic acid concentration reaches a predetermined value, the amount of the substrate-containing solution to be fed is decreased, or the feeding is ceased while the amount of the culture supernatant to be withdrawn with the culture medium supernatant pump 11 is decreased, or the withdrawal is ceased. Thus the lactic acid is assimilated.

The cultivation method of the present invention can be applied to the cultivation of cells capable of

producing useful substances, for example, monoclonal antibodies or lymphokines so as to efficiently produce said useful substances.

Animal cells secrete lactic acid which inhibits the proliferation of the cells. This acid is produced since the cells contain lactate dehydrogenase which produces lactic acid from pyruvic acid.

In the present invention, the lactic acid concentration in a culture medium is measured, and the amount of a substrate to be fed is controlled depending on the lactic acid concentration thus measured, based on the finding that animal cells, which are temporarily starved nutritionally, would assimilate lactic acid. When the lactic acid concentration falls below a predetermined value thereby the proliferation of the cells continues without being inhibited by lactic acid, which enables prolonged cultivation at a high cell density. Furthermore, the substrate to be added may be efficiently utilized thereby. Therefore, the method of the present invention is highly advantageous in the mass cultivation of animal cells.

To further illustrate the present invention, the following examples will be given with respect to Figs. 8 - 10.

In these examples, lactic acid secreted by cells was measured with an enzyme sensor, and the amount of the substrate to be fed was controlled to keep the lactic acid concentration in the culture medium below 2.5 g/l. 80 ml of a DM-180AU medium (mfd. by Kyokuto Seiyaku Kogyo K.K.) containing 10 % of newborn bovine serum was fed into a 200-ml tapping culture flask and then inoculated with seed cells of JTC-1 strain (rat ascites hepatoma cells) at a concentration of 4×10^5 cells/ml. Then the cells were cultured with stirring at 400 min^{-1} at 37°C in a CO_2 incubator. A 100 g/l aqueous solution of glucose was used as the substrate to be added. An appropriate amount of the glucose solution was fed everyday until the lactic acid concentration reached 2.5 g/l. When the lactic acid concentration reached 2.5 g/l, the amount of the substrate to be fed was decreased, or the feeding was ceased and the cultivation was continued. The lactic acid concentration and the glucose concentration were measured everyday before and after feeding the substrate. The cell concentration was measured with a hemacytometer. Fig. 10 shows the results. The lactic acid concentration reached 2.5 g/l after five days, and the proliferation stopped when the cell concentration reached 5.5×10^6 cells/ml. Then the amount of the glucose to be fed was decreased at the fifth day, and the feeding thereof was ceased at the sixth day. As a result, it was confirmed that the cell concentration increased with a decrease in the lactic acid concentration. These facts suggest that the cell concentration can be increased, and the

substrate-containing medium can be efficiently utilized by controlling the lactic acid concentration in the culture medium.

According to the present invention, the lactic acid concentration in a culture medium can be controlled in the cultivation of animal cells, which makes it possible to efficiently culture the animal cells at a high density while using less of the substrate-containing medium than the prior art, that is while efficiently using the substrate.

The present invention makes it possible to stably control cultivation conditions for animal cells for a prolonged period of time.

Claims

1. A method for controlling cultivation conditions for animal cells in a culture medium containing a substrate, characterized by

- measuring the lactic acid concentration (c_l) in the culture medium of the animal cell, and
- controlling the dissolved oxygen concentration and/or the substrate concentration (c_s) in the culture medium in accordance with the measured lactic acid concentration (c_l).

2. The method according to claim 1, characterized by

- measuring the lactic acid concentration (c_l) in the culture medium of the animal cells,
- measuring the substrate concentration (c_s) of the culture medium of the animal cells,
- determining the concentration (L) of lactic acid formed in a predetermined time,
- determining the concentration (S) of the substrate consumed in that predetermined time,
- calculating the ratio L/S ,
- and
- controlling the dissolved oxygen concentration and/or the substrate concentration (c_s) in the culture medium in accordance with the ratio L/S .

3. The method according to claim 1 or 2, characterized by

- measuring the lactic acid concentration (c_l) and the substrate concentration (c_s) of the culture medium of the animal cells at predetermined times defining predetermined time periods (Δt) or continuously,
- calculating the concentration (L) of lactic acid formed during the time periods (Δt) between subsequent predetermined times or continuously,
- calculating the concentration (S) of substrate consumed during the same predetermined time periods (Δt),
- calculating the respective ratio L/S ,
- and
- controlling the dissolved oxygen concentration

and/or the substrate concentration (c_s) in the culture medium such as to keep the ratio L/S within a predetermined, fixed range.

4. The method according to one of claims 1 to 3, characterized in that glucose is used as substrate.

5. The method according to one of claims 2 to 4, characterized in that the dissolved oxygen concentration and/or the substrate concentration (c_s) are controlled by stepwise supplying oxygen and/or substrate to the culture medium at predetermined times, and the ratio L/S is determined for each time period (Δt) between these predetermined times.

6. The method according to one of claims 2 to 5, characterized in that controlling the dissolved oxygen concentration and/or the substrate concentration (c_s) in the culture medium is performed when the ratio L/S is or tends to be outside the predetermined range.

7. The method according to one of claims 2 to 6, characterized in that the predetermined, fixed range of the ratio L/S is defined as to be $0 < L/S \leq A$, A being an upper limit value depending on the kind of the cultivated animal cells, and preferably being 0.6.

8. The method according to one of claims 2 to 7, characterized in that controlling is performed by increasing the dissolved oxygen concentration and/or by decreasing the glucose concentration (c_g) in the culture medium.

9. The method according to one of claims 1 to 8, characterized in that oxygen is supplied to the culture medium continuously for the entire culturing time.

10. The method according to one of claims 1 to 9, characterized in that during the entire culturing time, the animal cells are periodically separated from the culture medium and put into a new culture medium, preferably into a new culture medium having a higher substrate/glucose concentration than the used culture medium.

11. The method according to one of claims 1 to 10, characterized in that controlling is performed by reducing the substrate/glucose concentration to thereby reduce cell growth and induce lactic acid assimilation when the lactic acid concentration (c_l) of the culture medium reaches a predetermined value, preferably 2.5 g/l.

12. The method according to one of claims 1 to 11,

characterized in that throughout the cultivation substrate/glucose is added until the lactic acid concentration (c) reaches the predetermined value.

13. The method according to one of claims 1 to 12, characterized in that controlling is performed by reducing the substrate/glucose concentration by controlling or by stopping the addition of substrate/glucose.

14. The method according to one of claims 1 to 13, characterized by the use of animal cells of a type expressing a gene of an enzyme which assimilates lactic acid, particularly a cardiac type lactate dehydrogenase.

15. The method according to one of claims 1 to 14, characterized in that animal cells originating from liver are used.

16. An apparatus for controlling cultivation conditions for animal cells in a culture medium containing a substrate, particularly for carrying out the method according to one of claims 1 to 15, comprising

- a fermentor (1) for the culture medium (2) provided with a stirrer (4),

- a substrate tank (5) connected to the fermentor (1) by means of a conduit (14) comprising a control valve (7),

- a cell/culture medium separator (10) provided at the discharge side of the fermentor (1),

- a culture medium supernatant pump (11) provided in the discharge conduit (17) of the fermentor (1),

- lactic acid concentration sensing means (9) provided at or in the fermentor (1), and

- an operation controller (12) for controlling the control valve (7) and receiving the output signal of the lactic acid concentration sensing means (9).

17. The apparatus according to claim 16, further comprising

- means (6, 13) for supplying oxygen to the fermentor (1),

- and
- glucose concentration sensing means (8) provided at or in the fermentor (1) and connected to the operation controller (12).

18. The apparatus according to claim 16 or 17, characterized in that the oxygen supply means comprise an oxygen supply conduit (13) and a control valve (6) provided in the conduit (13) and being controlled by the operation controller (12).

19. The apparatus according to one of claims 16 to 18, characterized in that the operation controller (12) is adapted to carry out the method according to one of claims 1 to 15 by controlling the supply of

oxygen and/or substrate by means of the control valves (6) and/or (7), and eventually the discharge of the culture medium and the separation of the culture medium supernatant by means of the cell/culture medium separator (10), on the basis of the output signals of the lactic acid concentration sensing means (9) and eventually also the glucose concentration sensing means (8).

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Fig. 1a

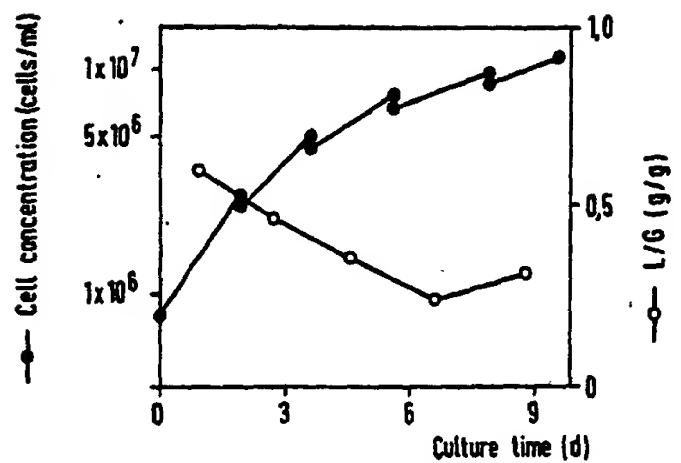


Fig. 1b

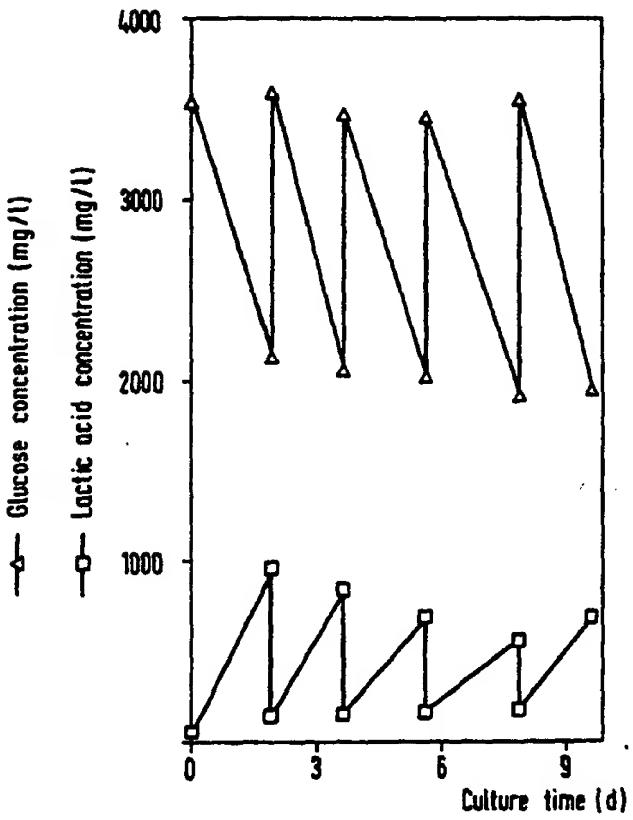


Fig. 2a

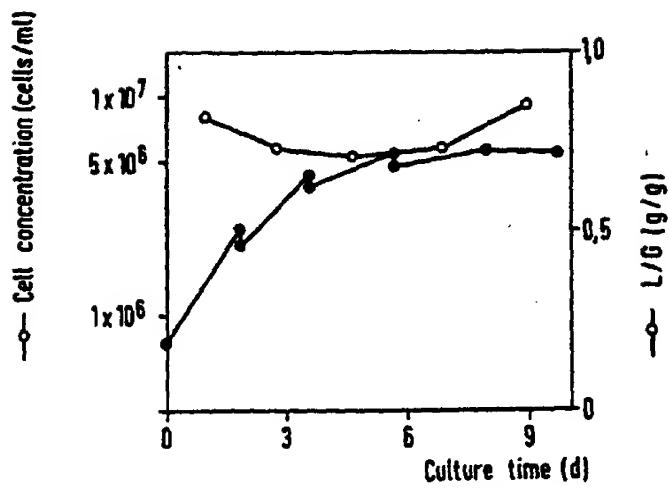


Fig. 2b

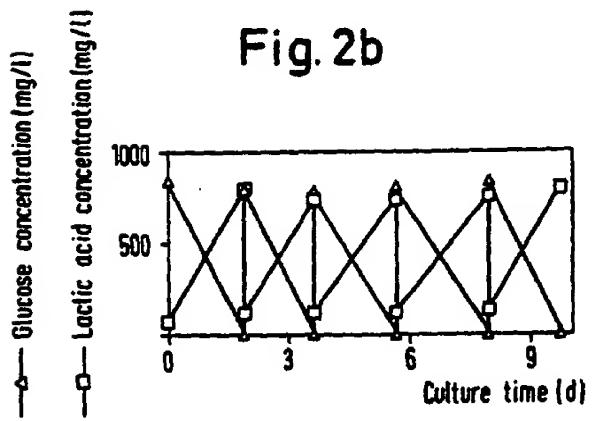


Fig. 3a

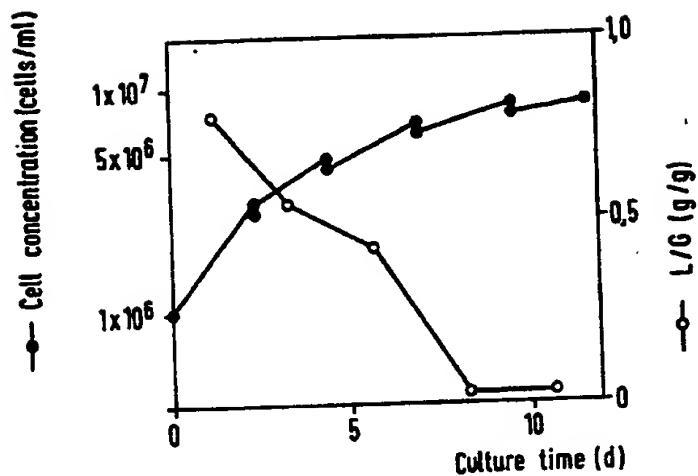
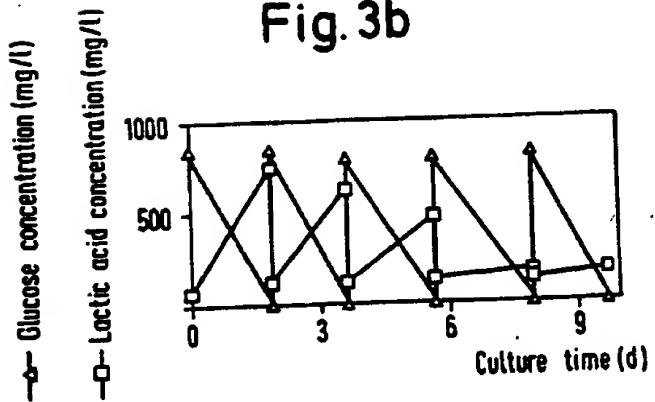


Fig. 3b



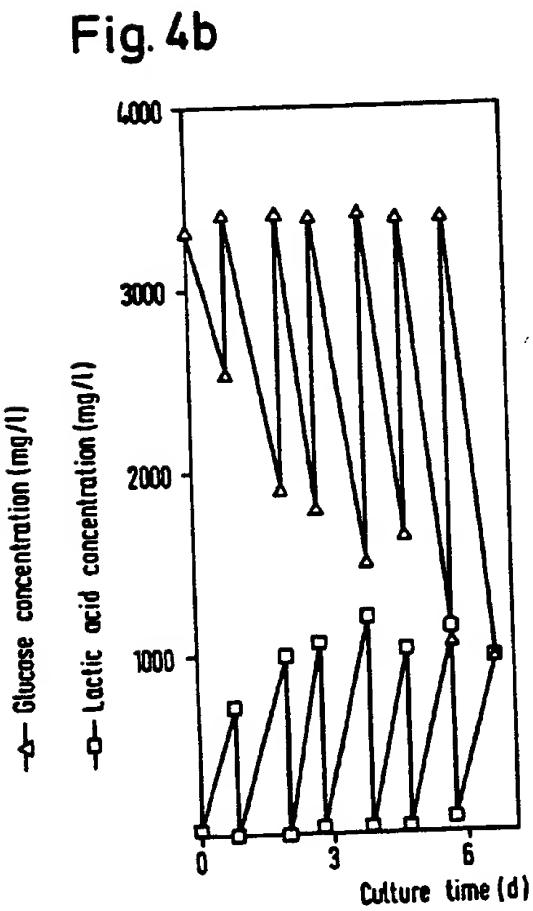
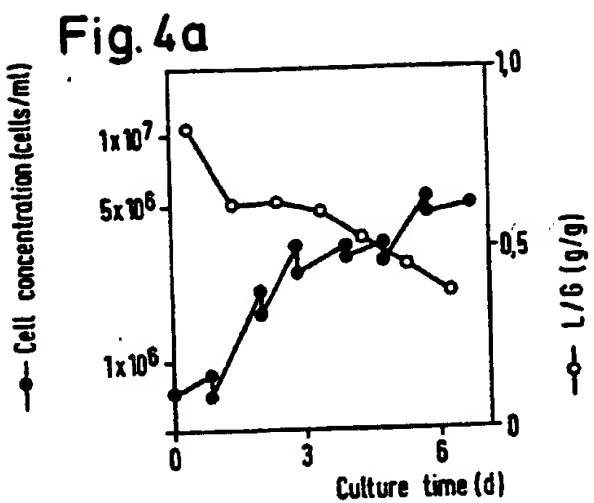


Fig. 5a

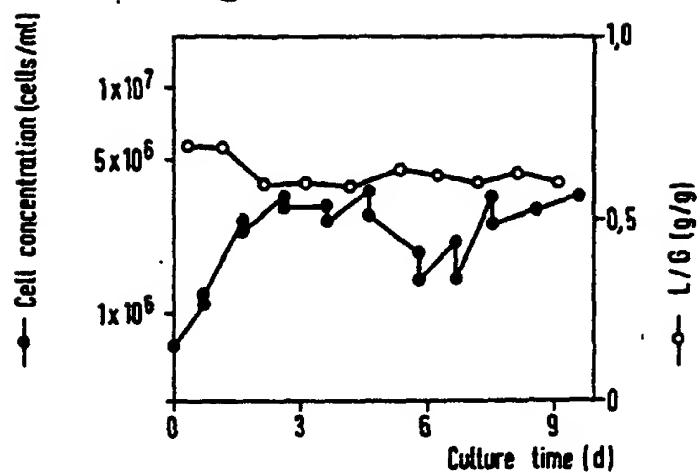


Fig. 5b

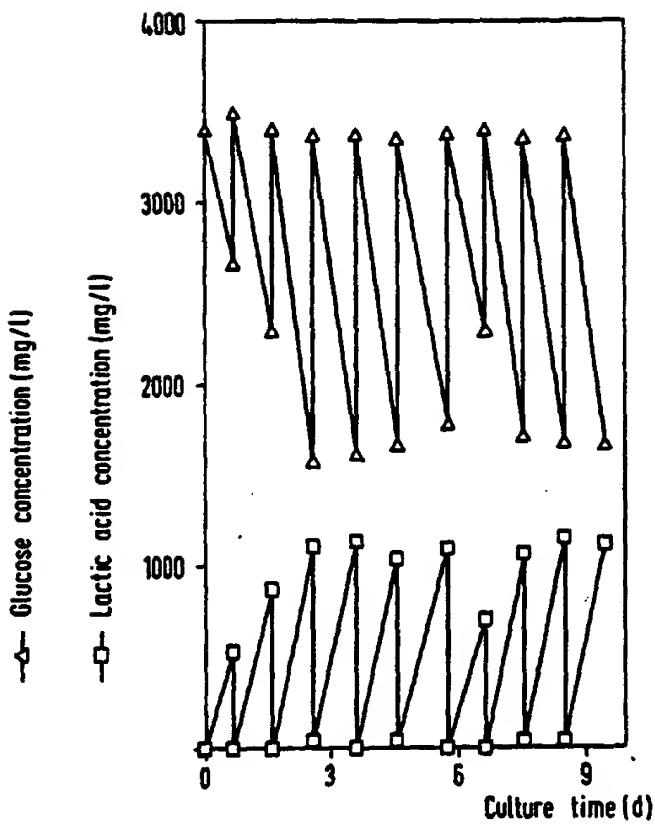


Fig. 6

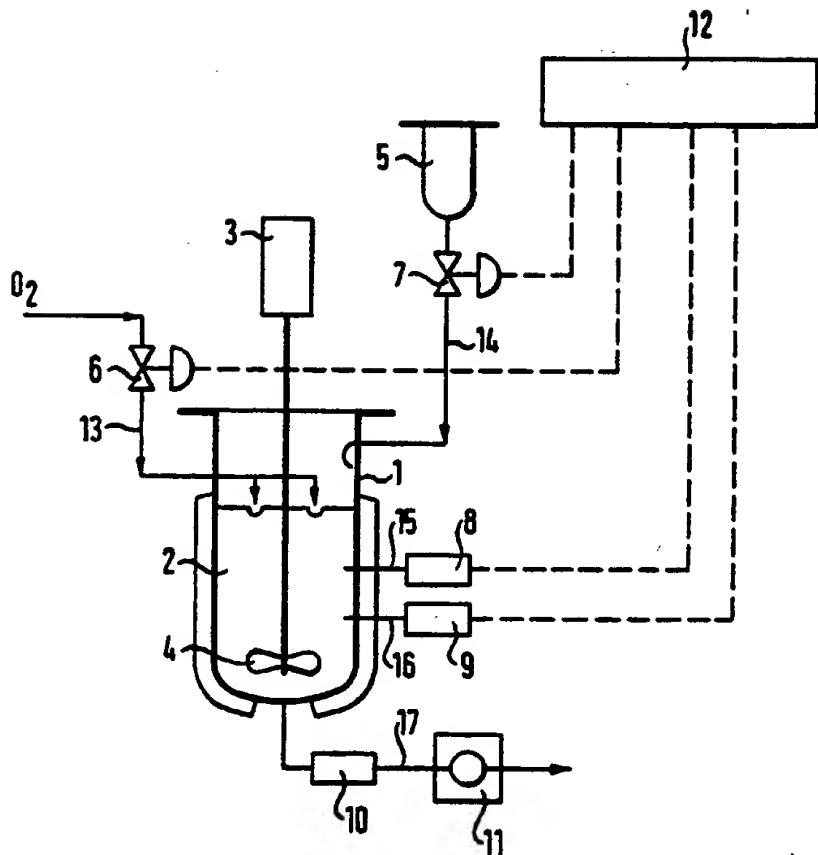


Fig. 7a

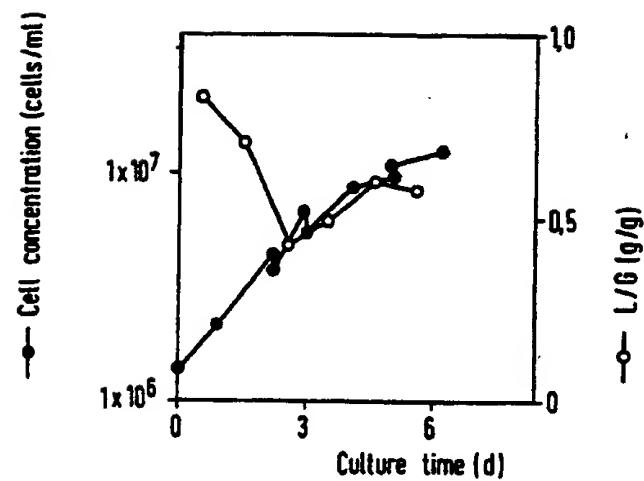


Fig. 7b

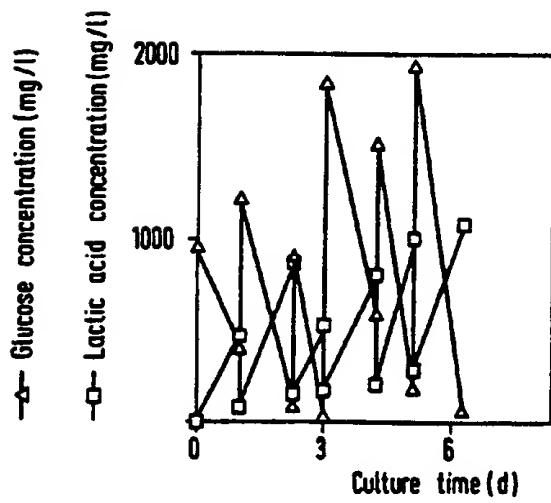


Fig. 8a

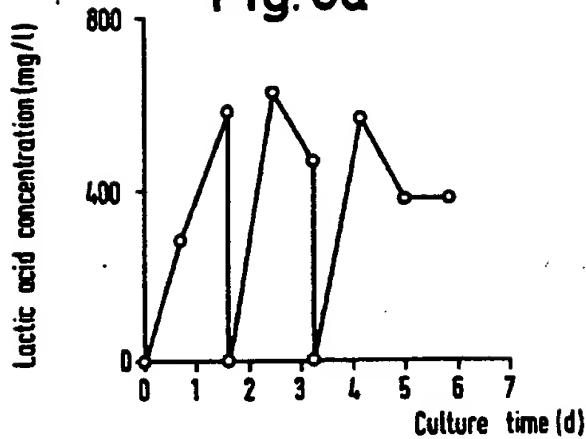


Fig. 8b

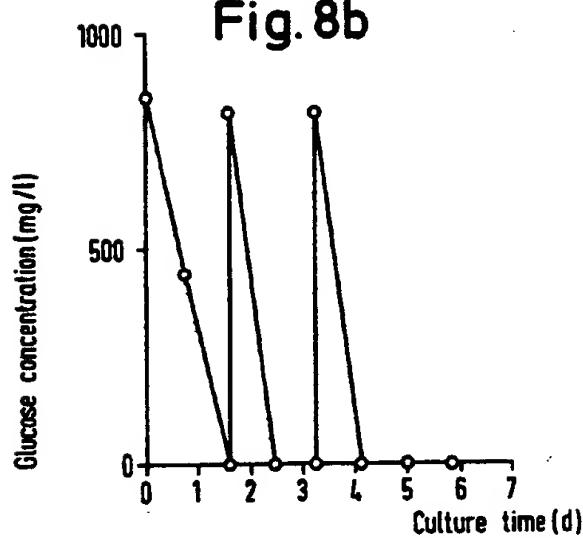


Fig. 8c

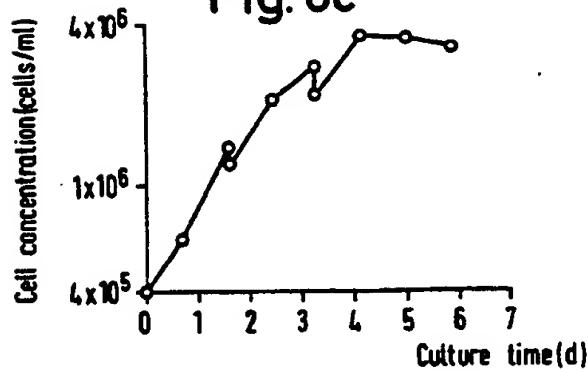


Fig. 9

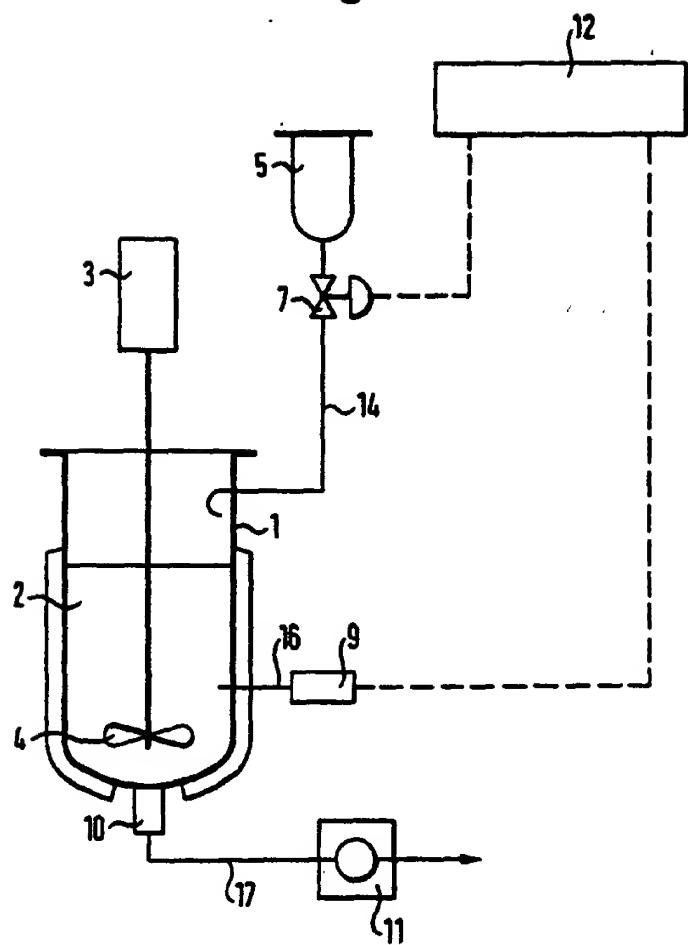


Fig. 10a

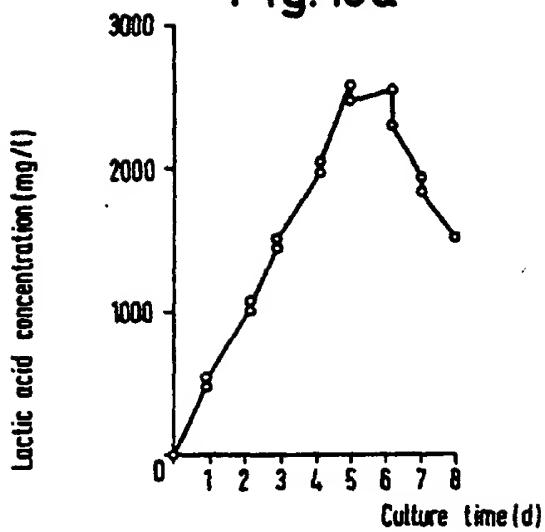


Fig. 10b

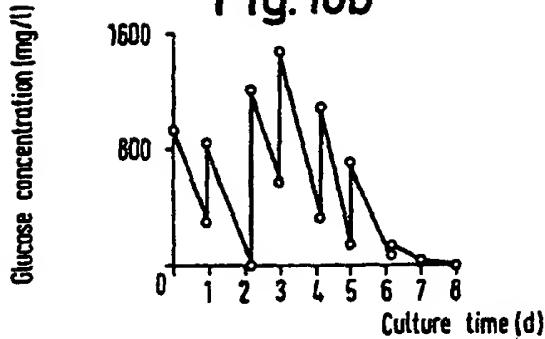
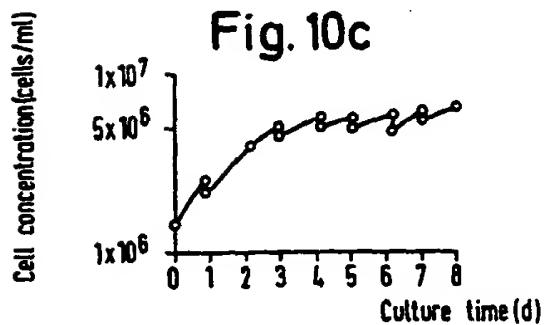


Fig. 10c





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EUROPEAN SEARCH REPORT

Application Number

EP 90 10 4822

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y	BIOTECHNOLOGY & BIOENGINEERING, vol. 27, no. 6, June 1985, pages 837-841, New York, US; T. TSUCHIDA et al.; "Application of L-(+)-lactate electrode for clinical analysis and monitoring of tissue culture medium" * Page 840, column 2, paragraph 1; page 841, column 1, paragraph 4 *	1-19	C 12 M 1/36 C 12 N 5/00
A	FR-A-2 578 266 (NEW BRUNSWICK SCIENTIFIC CIE, INC.) * Claims *	1	
A	DE-A-2 657 209 (BENDER & HOBIN GmbH) * Claim 1 *	1	
A	GB-A-1 090 758 (THE WELLCOME FOUNDATION LTD) * Page 1, lines 27-41; claims 1,2 *	1	
Y	WO-A-8 801 643 (ENDOTRONICS, INC.) * Claim 1; page 3, line 1 - page 8, line 4 *	1-19	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
Y	PATENT ABSTRACTS OF JAPAN, vol. 13, no. 29 (C-562)[3377], 23rd January 1989; & JP-A-63 233 780 (HITACHI LTD) 29-09-1988 * Abstract *	1-19	C 12 M C 12 N
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	26-06-1990	COUCKE A.O.M.	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
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